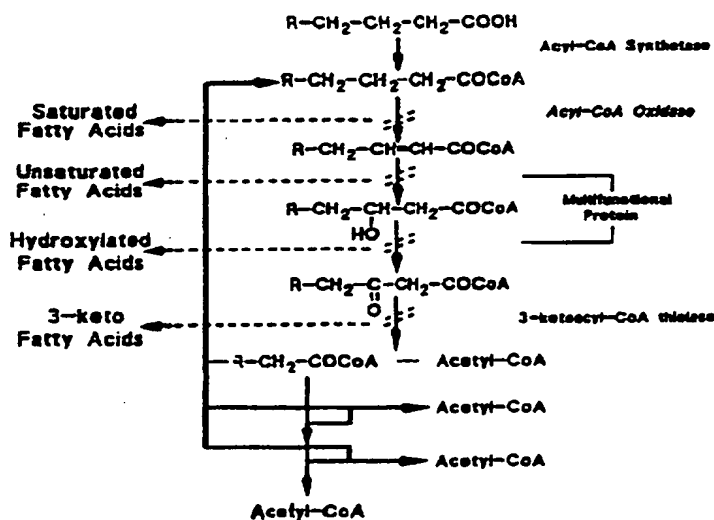




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(54) Title: TRANSGENIC ANTISENSE-PLANTS SHOWING REDUCED ENZYMIC ACTIVITY IN BETA-OXIDATION



Pathway of β -oxidation showing end-products formed from a reduced or missing enzymatic activity (broken lines and arrows).

(57) Abstract

Nucleotide sequences encoding antisense RNA to proteins having enzymic activity in β -oxidation, constructs, vectors, plants and plant cells comprising such nucleotide sequences, and uses of such nucleotide sequences.

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TRANSGENIC ANTISENSE-PLANTS SHOWING REDUCED ENZYMIC ACTIVITY IN BETA-OXIDATION**Field of Invention**

The present invention relates to cells exhibiting a lower rate of the β -oxidation of fatty acids, processes for obtaining such cells, and genetic material therefor. In particular, the cells are plant cells.

Crop plants, such as oilseed rape, sunflower and corn, are valuable agronomically as sources of oils which can be used for many purposes ranging from use as industrial feed stocks to use in margarine manufacture and even as potential alternatives to fossil fuels.

The production of naturally occurring oils in oil producing plants has been augmented hitherto using conventional breeding techniques. Generally speaking, it is the seeds of oil producing plants which are harvested and then processed for their oil content, the rest of the plant generally being left as waste.

Recently, it has been proved that recombinant DNA technology can be used, for example, on oil seed rape to produce fatty acids which are not found naturally in the non-transformed plant. Voelker et al. Plant Journal (1996) 9: 229-241 succeeded in engineering lauric acid production into oil seed rape. Lauric acid is a fatty acid not normally found in any significant quantity in oil seed rape. Although, the production of lauric acid in oil seed rape was achieved, it has been found that the presence of lauric acid also plays a part in activating fatty acid catabolism (β -oxidation pathway), thus creating a so-called

"futile cycle" wherein lauric acid is produced which in turn plays a role in initiating its own catabolism, being degraded and resulting in decreased yields of lauric acid in such plants (Eccleston V.S. et al. *Planta* (1996) 198: 46-53).

There exists a need to improve the overall yield of fatty acids and/or lipids in oil seed bearing plants. For the purposes of the description oil bearing plants are to be construed as plants which are agronomically attractive for their fatty acid and/or lipid generating potential and/or capacity. In particular, there exists a need to modify the β -oxidation pathway in plants, thereby improving overall yield of naturally occurring fatty acids or non-naturally occurring fatty acids (i.e. fatty acid production as a result of recombinant DNA manipulation) in oil seed bearing plants.

An object of the present invention is to provide plants, in particular oil seed bearing plants with improved yields of oils in the seeds.

A second object of the invention is to improve fatty acid and/or lipid levels in plant tissues other than seeds.

These and other objects of the invention will become apparent from the following description and examples.

According to the present invention there is provided a nucleotide sequence comprising a transcriptional regulatory sequence, a sequence under the transcriptional control thereof which encodes an RNA which consists of a plurality of subsequences characterised in that the RNA subsequences are anti-sense RNA's to mRNA's of proteins having an enzymic activity in the peroxisomal β -oxidation of fatty acids in plant cells.

The nucleotide sequence encoding the antisense RNA molecule and consisting of a plurality of subsequences can be of any length provided that the antisense RNA molecule transcribable therefrom is sufficiently long so as to be able to form a complex with a sense mRNA molecule encoding for at least a protein having an enzymic activity in the β -oxidation pathway. Thus, without the intention of being bound by theory it is thought that the antisense RNA molecule complexes with the mRNA of the protein or proteins and prevents or substantially inhibits the synthesis of functional protein(s) having enzymic activity in the β -oxidation pathway. As a consequence of the interference of the antisense RNA enzyme activity of protein(s) involved in the β -oxidation of fatty acids is decreased.

For the purposes of the description "nucleotide sequence" will be referred to as DNA unless there is different indication. The DNA encoding the antisense RNA can be from about 50 nucleotides in length up to the length of the relevant mRNA produced by the cell. Preferably, the length of the DNA encoding the antisense RNA will be from 100 to 1500 nucleotides in length. The preferred source of antisense RNA for DNA constructs of the present invention is DNA showing substantial identity or similarity to the genes or fragments thereof of proteins having enzymic activity involved in the three major steps of β -oxidation in plants. Thus the encoding DNA of constructs of the present invention may be selected from the group acyl-CoA oxidases, for example long chain acyl CoA (C_{16} -CoA), medium chain acyl CoA (C_{12} -CoA) and/or short chain acyl CoA (C_6 -CoA) oxidases (Hooks et al. (1995) *Phytochemistry* 40, p. 657), multi-functional protein and

3-ketoacyl-CoA thiolase or fragments thereof such as enzymically active fragments thereof.

The promoter is a nucleotide sequence upstream from the transcriptional initiation site and which contains all the regulatory regions required for transcription. Examples of promoters suitable for use in DNA constructs of the present invention include viral, fungal, bacterial, animal and plant derived promoters capable of functioning in plant cells. The promoter may be selected from so-called constitutive promoters or inducible promoters. Examples of suitable inducible or developmentally regulated promoters include the napin storage protein gene (induced during seed development), the malate synthase gene (induced during seedling germination), the small sub-unit RUBISCO gene (induced in photosynthetic tissue in response to light), the patatin gene highly expressed in potato tubers and the like. Alternatively, the promoter could be selected to express the DNA constitutively, that is, in all living tissues of the plant. It will be appreciated that the promoter employed should give rise to the transcription of a sufficient amount of the antisense RNA molecule at a rate sufficient to cause an inhibition of fatty acid catabolism in plant cells. The required amount of antisense RNA to be transcribed may vary from plant to plant. Examples of suitable promoters include the cauliflower mosaic virus 35S (CaMV 35S) and 19S (CaMV 19S) promoters, the nopaline synthase promoter, octopine synthase promoter, heat shock 80 (hsp 80) promoter and the like. Generally, in plants and plant cells of the invention inducible or developmentally regulated promoters are preferred.

A terminator is contemplated as a DNA sequence at the end of a transcriptional unit which signals termination of transcription. These elements are 3'-non-translated sequences containing polyadenylation signals which act to cause the addition of polyadenylate sequences to the 3' end of primary transcripts. Sequences mentioned above may be isolated from fungi, bacteria, animals or plants.

Examples of terminators particularly suitable for use in nucleotide sequences and DNA constructs of the invention include the nopaline synthase polyadenylation signal of *Agrobacterium tumefaciens*, the 35S polyadenylation signal of CaMV, octopine synthase polyadenylation signal and the zein polyadenylation signal from *Zea mays*.

The skilled addressee will appreciate that nucleotide sequences as defined herein may be introduced to plant cell genomes of oil bearing plants already transgenic for oils which do not occur naturally in native, non-transformed plants. An example of a plant of this type is *Brassica napus* which is transgenic for lauric acid production. Nucleotide sequences of the invention can be introduced into the plant cell genome of such plants. It is thought that such introduced antisense nucleotide sequences of the invention will reduce futile recycling of non-naturally occurring fatty acids produced in plants genetically transformed for the production thereof.

The nucleotide sequence may encode an RNA having any number of subsequences. Preferably, the number of subsequences lies between 2 and 7 (inclusive) and more preferably lies between 2 and 4.

In one preferment, the RNA encoded by the contiguous sequence comprises a cleavage site, such as a ribozyme or restriction enzyme site such as XbaI, SalI, KpnI or the like, between two of the subsequences so that the RNA can be cleaved into regions comprising said subsequences, or even into the subsequences per se. Naturally, the skilled addressee will appreciate that the subsequences contained within the RNA encoded by the contiguous sequence resulting from such cleavage will not contain a 5' cap or a ribosome binding site and will thus not be translated when present in a eukaryotic cell, such as a plant cell.

The invention still further provides a nucleotide sequence which is similar to the above disclosed antisense RNA sequences. By "similar" is meant a test sequence which is capable of hybridising to a sequence which is complementary to the inventive nucleotide sequence. When the test and inventive sequences are double stranded the nucleic acid constituting the test sequence preferably has a T_m within 20°C of that of the inventive sequence. In the case that the test and inventive sequences are mixed together and denatured simultaneously, the T_m values of the sequences are preferably within 10°C of each other. More preferably the hybridization is performed under stringent conditions, with either the test or inventive DNA preferably being supported. Thus either a denatured test or inventive sequence is preferably first bound to a support and hybridization is effected for a specified period of time at a temperature of between 50 and 70°C in double strength SSC ($2\times\text{NaCl}$ 17.5g/l and sodium citrate (SC) at 8.8g/l) buffered saline containing 0.1%

sodium dodecyl sulphate (SDS) followed by rinsing of the support at the same temperature but with a buffer having a reduced SSC concentration. Depending upon the degree of stringency required, and thus the degree of similarity of the sequences, such reduced concentration buffers are typically single strength SSC containing 0.1%SDS, half strength SSC containing 0.1%SDS and one tenth strength SSC containing 0.1%SDS. Sequences having the greatest degree of similarity are those the hybridization of which is least affected by washing in buffers of reduced concentration. It is most preferred that the test and inventive sequences are so similar that the hybridization between them is substantially unaffected by washing or incubation in one tenth strength sodium citrate buffer containing 0.1%SDS.

The invention still further provides a nucleotide sequence which is complementary to one which hybridizes under stringent conditions with the above disclosed nucleotide sequences.

The invention also provides a DNA construct comprising the nucleotide sequence according to the invention, as well as a biological vector comprising the said sequence or construct. The biological vector may be a virus or bacterium, such as *Agrobacterium tumefaciens*, for example, and the construct advantageously further encodes a marker protein, such as one having herbicide resistance, or anti-bacterial properties.

DNA constructs and nucleotide sequences of the invention may be used to transform cells of both monocotyledonous and dicotyledonous plants in various ways known in the art. For example, particle bombardment of embryogenic callus is the method of choice for production of transgenic monocotyledonous plants

[Vasil (1994) *Plant Mol. Biol.* 25, 925-937]. In many cases transformed plant cells may be cultured to regenerate whole plants which can subsequently reproduce to give successive generations of genetically modified plants.

The invention still further provides eukaryotic cells, such as plant cells (including protoplasts) for example, containing the said nucleotide sequence, DNA construct or vector.

The invention still further provides plants comprising such plant cells, the progeny of such plants which contain the sequence stably incorporated and heritable in a Mendelian manner, and/or the seeds of such plants or such progeny. Such plants include field crops including sunflower, oilseed rape, soybean, castorbean, maize, olive, linseed and cuphea. Plants and/or plant cells of the *Brassicaceae* are particularly preferred, such as oilseed rape and *Arabidopsis*. By "modified fatty acid content" is meant a cell which exhibits non-wild type proportions of fatty acids and/or lipids due to under-expression of an enzymic protein of β -oxidation.

The invention still further provides the use of the sequence according to the invention, whether "naked" or present in a DNA construct or biological vector - in the production of eukaryotic cells, particularly plant cells having a modified fatty acid content.

The invention still further provides a method of inducing an under expression of an enzymic protein of β -oxidation in plant cells comprising introducing into such cells a nucleotide sequence according to the invention, or a construct or vector containing it.

The invention still further provides a method of inhibiting the production of at least one enzyme in a eukaryotic cell comprising introducing into the said cell a nucleotide sequence comprising a transcriptional regulatory sequence and a sequence contiguous therewith and under the transcriptional control thereof, which contiguous sequence encodes an RNA which consists of a plurality of subsequences, characterized in that the subsequences have the sequences of antisense RNA's to mRNA's of proteins having an enzymic activity in the peroxisomal β -oxidation of fatty acids in a plant.

Examples of the nucleotide sequences of the invention are provided below. These examples relate to the production of plants of the family *Brassicaceae* such as oil seed rape and *Arabidopsis*.

1. The nucleotide sequence of the invention may encode an mRNA which consists - in the 5' to 3' direction - of (i) a promoter, (ii) at least two cDNA's in reverse orientation i.e. 3' to 5' orientation, (iii) a terminator, (iv) optionally a further promoter, (v) the coding region of the HPT II gene (hygromycin) and (vi) optionally a further stop codon. When such a sequence is introduced into the cells of *Brassicaceae* plants, the sequence encoding the mRNA is transcribed. The region of the thus transcribed mRNA which encodes the HPT II gene is translated, whilst the region of the mRNA which encodes the at least two cDNA's is not.

2. The nucleotide sequence of the invention may encode an mRNA which consists - in the 5' to 3' direction - of (i) a promoter, (ii) the coding region of the HPT II gene, (iii) a translation stop codon, (iv) optionally a further start codon, (v) a region encoding at least two cDNA's in reverse orientation i.e. 3' to 5' orientation and (vi) optionally a further stop codon. When such a sequence is introduced into the cells of *Brassicaceae* plants, the sequence encoding the mRNA is transcribed. The region of the thus transcribed mRNA which encodes the HPT II gene is translated, whilst the region of the mRNA which encodes the cDNA's in reverse orientation, i.e. 3' to 5' orientation is not translated.

3. The nucleotide sequence of the invention may encode an mRNA which comprises in the 5' to 3' direction (i) a promoter, (ii) a cDNA in reverse orientation i.e. 3' to 5' orientation, (iii) a terminator, (iv) a promoter, (v) the coding region of the HPT II gene (hygromycin), (vi) a terminator, (vii) a promoter, (viii) a second cDNA in reverse orientation i.e. 3' to 5' orientation, (ix) a terminator. When such a sequence is introduced into the cells of *Brassicaceae* plants, the sequences encoding (ii) and (viii) are transcribed. The region of the thus transcribed mRNA which encodes the HPT II gene is translated, whilst the regions of the mRNA encoding the cDNA is not.

Naturally the skilled addressee will also appreciate that the two (2) (or more) cDNA's in reverse orientation could be adjacent to one another and located upstream or downstream of a marker gene, if present.

The invention will now be described with reference to the following Figures and Examples which are not to be construed as limiting the invention.

Figure 1: Pathway of β -oxidation showing end-products formed with a reduced or missing enzymatic activity (broken lines and arrows).

Figure 2A & 2B: Fatty acids and ACOX activities in *Arabidopsis* (EMS) mutants requiring exogenous carbohydrate for early post-germinative growth.

Figure 3A & 3B: ACOX activity in *Arabidopsis* during two different developmental states.

Figure 4A & 4B: Maps of transformation vectors.

Figure 5: Map of double antisense vector.

Figure 6: Southern blots showing incorporation of cDNA into the genome of *Arabidopsis*.

Figure 7A & 7B: Dependence of early post-germinative growth of ACOX antisense seedlings on exogenous carbohydrate.

Figure 8A & 8B: Reduced ACOX activity in antisense lines.

Figure 9: Induction of genes of fatty acid catabolism in developing seeds of plants genetically engineered to produce lauric acid.

Example 1: Fatty Acid Contents and ACOX Activities in *Arabidopsis* Mutants that Require an Exogenous Carbohydrate Source for Germination and Growth

A number of EMS mutagenised seed lines accumulate various different non-polar lipids that are not present in wild type plants. *Arabidopsis* seedlings requiring an exogenous carbohydrate supply for growth were selected from a population

of M2 seed ultimately derived from ethylmethylenesulfonic acid (EMS) mutagenized Col-0 seed. The general procedures for mutagenizing seed with EMS and selecting for mutants are described in detail in *Arabidopsis* (Meyerowitz & Somerville, eds., Cold Spring Harbor Laboratory Press, 1994). The phenotype of these mutants is a drastically reduced growth compared to wild-type Col-0 when grown on agar plates containing 1/2 MS salts, but no carbohydrate. The growth of mutant seedlings in the presence of exogenous carbohydrate is similar to that of wild-type Col-0.

All seeds were germinated in petri dishes on a 25 ml, 0.8% agar base containing 1/2 concentration of Murashige and Skoog salts (1/2 MS salts) [Murashige and Skoog (1962) *Physiol. Plant* 15, 473], 1x Gambourg's B5 vitamins, and with or without sucrose. All seeds were surface sterilized by soaking for 8 minutes in a solution of 50% sodium hypochlorite containing 0.05% Triton X-100 and left in H₂O at 4°C for at least 24 hours before being plated under sterile conditions in a flow hood.

Lipid contents of mutant seeds, both endogenous and in storage lipids, were determined by thin-layer chromatography of lipid extracts isolated according to standard protocols [Kates (1986) *Techniques of lipidology: Isolation, Analysis, and Identification of Lipids*, 2nd ed. Elsevier Press, Amsterdam]. In each case the lipids from 5 seeds or 5-day old plantlets from 5 seeds were extracted, resuspended each in the same volume of chloroform and chromatographed on silica gel plates using hexane/ether/acetic acid (90:10:1) as the running solvent. The lipids were visualized by spraying the plates with concentrated

H₂SO₄ and heating them at 160°C for a few minutes. An example of a mutant containing a new lipid in seeds and one exhibiting the accumulation of an aliphatic compound during post-germinative growth are shown in Figure 2A.

Mutants exhibiting the sucrose-dependent-growth phenotype due to disrupted β -oxidation would also be expected to exhibit reduced ACOX activity, either due to a mutation in an ACOX gene or because ACOX activity is directly tied to the flux through β -oxidation (Chu et al. (1994) *Biochem. J.* 302, 23]. Therefore, groups of 100 4-day old mutant seedlings were analyzed for ACOX activity as specified in Example 2. The mutants analyzed exhibited reduced ACOX activity with C₁₀CoA (MCOX) to a certain degree (Figure 2B).

Example 2: **Identification of Three Acyl-CoA Oxidases in the Brassicaceae Species *Arabidopsis Thaliana***

Previous work by one of the authors has shown that three acyl-CoA oxidases exist in the monocotyledonous species *Zea Mays*. The basis for their identification was the different relative levels of ACOX activity, determined with a short-(C₆CoA), a medium- (C₁₀CoA), and a long-chain (C₁₆CoA) acyl-CoA substrate, that existed in maize tissues of various development and metabolic states [Hooks et al. (1994) *Plant Physiol.* 105, s710; Hooks et al. (1995) *Phytochemistry* 40, 657]. A similar analysis of ACOX activity in *Arabidopsis* during post-germinative growth on senescence, using the acyl-CoA substrates preferred by each maize enzyme, has shown the three corresponding ACOXs, LCOX, MCOX, and SCOX to exist in the dicotyledonous species,

Arabidopsis thaliana. All protein extraction and ACOX activities measurements were done according to the method of Hooks et al (1995) *Phytochemistry* 40, 657.

For the analysis of ACOX activity in *Arabidopsis* seedlings, Col-0 seeds were germinated on agar plates containing 1/2 MS salts and 20 mM sucrose. Each day for eight days 100 seedlings were taken, the fresh tissue weight recorded, and then each group of 100 seedlings was extracted and assayed for ACOX activity with the three substrates. Different relative levels of ACOX activity with the three different substrates is apparent (Figure 3A).

For the analysis of ACOX activity in senescing leaves of *Arabidopsis*, leaves from mature Col-0 plants were excised and incubated in the dark for one to 10 days on moist filter paper. Proteins were extracted from the leaves and ACOX activities measured with the three substrates. A different time course of induction and diminution of ACOX activity with each of the three substrates is again apparent (Figure 3B). The profiles of ACOX activity were compared to the glyoxylate cycle enzyme isocitrate lyase (ICL), an enzyme known to be induced during the senescence of leaves. ICL activity is generally regarded as an indicator of high degrees of fatty acid catabolism.

Example 3: **Identification of cDNA Sequences Coding for Enzymes of β -Oxidation**

All cDNA clones were obtained from the *Arabidopsis* Biological Resource Center, DNA Stock Center (1060 Carmack Rd. Columbus, OH 43210-1002). The cDNA clones for the acyl-CoA oxidases (35H7T7 & 5F12T7P), and 3-ketoacyl-CoA thiolase (39H3T7)

were provided as *E. coli* bacterial colonies containing the cloning vector λ -Ziplox (GIBCO BRL) into which each cDNA clone was directionally inserted 5' to 3'. The cDNA clone for the multifunction protein, FAFJ01, was provided as an *E. coli* bacterial colony containing the clone directionally inserted 5' to 3' into λ -ZAP II (Stratagene). The sequences for each clone, determined by partial sequencing of the 5' end [Newman, et al., (1994) *Plant Physiol.* 106, 1241-1255], are given below. Each cDNA clone was identified by sequence comparison to known β -oxidation enzymes using the tBlastn program available by electronic mail at blast@ncbi.nlm.nih.gov, and scored with the default values of the algorithm. The score indices given below for each cDNA are generally regarded as proof of their identification [Altschul et al. (1990) *J. Mol. Biol.* 215, 403]. The sequence of each cDNA clone provided was confirmed by us using standard sequencing techniques.

Acyl-CoA Oxidase:**DEFINITION** 519 Arabidopsis thaliana cDNA clone 35H7T7.**ACCESSION** T04472

```

1   ATTGAGACAC AGGTGATTGA TTATAAACT CAGCAGAACA GGCTATTTCC
51  TCTGCTAGCA TCTGCATATG CATTTCGATT TGTTGGAGAG TGGCTAAAAT
101 GGCTGTACAC GGATGTAACT GAAAGACTGG CGGCTAGTGA TTTCGCAACT
151 TTGCCTGAGG CTCATGCATG CACTGCAGGA TTGAAGTCTC TCACCACCAC
201 AGCCACTGCG GATGGCATTG AAGAATGTCT TAAGTTATGT GGTGGACATG
251 GATACTTGTC GTGCAGTGGG CTCCCCGAGC TGTTTGCTGT ATATGTTCCCT
301 GCCTGCACAT ACGAAGGAGA CAATGTTGTG CTGCAATTAC AGGTTGCTCG
351 ATTCCTCATG AAGACAGTCG CCCAGCTGGG ATCTNGAAAG GTTCNGTTTG

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wherein N = A, C, G or T

DEFINITION 858 Arabidopsis thaliana cDNA clone 5F12T7P.**ACCESSION** T04810

```

1   GTGCGTTGAG ATTCCGTTCT GTGAGAATAC CCCGTGATAA TCTTCTCAAT
51  CGTTTTGGAG ATGTGTCCCG AGATGGGACG TATACAAGTA GTTTGCCAAC
101 AATCAATAAA AGATTTGGTG CAACACTCGG TGAGCTTGTA GGTGGTCGAG
151 TTGGCCTTGC CTATGCATCT GTTGGCGTCC TTAAAATCTC TGCAACGATT
201 GCCATTGCGT ATTCTCTTCT AAGACAACAA TTCGGGCCTC CAAAGCAACC
251 TGAGGTCAGT ATTCTCGATT ACCAGTCTCA ACAACACAAG CTCATGCCGA
301 TGTTAGCCTC CACCTATGCA TACCATTTTG CAACTGTATA CCTTGTGGGG
351 GAATATTGAG AGATGAAGAA GGCTCACGGT GAGCAATTGG TTGCTGATGT
401 CCATGCACTC TCTGCTGGGC TCAAATCTTT GTACGGGTTT CACGCAGGGT
451 CTCGCCTTAG AAAGCTTTTG

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TBlastN of dBest

Query = Rat ACOX j02752 (661 letters)

reading High Probab.
Frame Score P(N) N

Sequences producing High-scoring Segment Pairs:

35H7T7

gnl/dbest/21287 T04472 cDNA Lambda-PRL2 A. thaliana H... +1 380 5.2e-45 1

5F12T7P

gnl/dbest/21625 T04810 cDNA Lambda-PRL2 A. thaliana H... +3 120 1.5e-20 3

Multifunctional Protein:

DEFINITION A. thaliana transcribed sequence; clone FAFJ01
ACCESSION Z31666

1	CCTGGTGGGA	AGCCTATATC	AGTACCTGAT	AAAGAAATTG	TAGAGATGAT
51	CTTATTCCCT	GTTGTCAACG	AGGCATGCCG	CGTCCTAGAT	GAAGGAGTTG
101	TGATCCGAGC	CTCAGACTTG	GACATTGCGT	CTGTCCTTGG	AATGAGTTT
151	CCTTCTTACC	GGGGAGGAAT	TGTTTTCTGG	GCAGACACTG	TTGGCCCCAA
201	GTACATATAT	GAGAGGCTCA	AGAAATTGTC	GGAGACTTAT	GGCAGCTTTT
251	TCAMACCATC	GAGGTATCTG	GAGGAAAGAG	CAATGCAATG	GAATGCTTTT
301	TGAGTGAATC	GAAATCGTCG	AGGTCCACAT	TGTGACGGCG	TTTCA

wherein M = A or C.

TBlastN of dBest

Query = Cucumber MFPx78996 (725 letters)

	reading	High	Probab.
Frame	Score	P(N)	N

Sequences producing High-scoring Segment Pairs:

gnl/dbest/44380 Z31666 cDNA Strasbourg-A A. thaliana ... +1 328 8.1e-38 1

3-ketoacyl-CoA thiolase (thiolase):

DEFINITION 442 Arabidopsis thaliana cDNA clone 39H3T7.
ACCESSION T04395

1	AACAGATCCG	AATTTTATCT	TTAATCAGCC	GGAAAAAATG	GAGAAAGCGA
51	TCGAGAGACA	ACNGTTCTT	CTTGAGCATC	TCCGACCTTC	TTCTTCTTCT
101	TCGCACAATT	ACGAGGCTTC	TCTATCTGCT	TCTGCTTGCT	TGGCTGGGGA
151	CAGTGCTGCA	TATCAGAGGA	CCTCTCTCTA	TGGAGATGAT	GTTGTCATTG
201	TCGCGGCACA	TAGGNCTCCA	CTATGCAAGT	CCAAACGTGG	CAATTTCAAG
251	GGTACATATC	CCGCTGATTT	GCTCGCACCT	NTTTTGAGGG	CATTGATAGA
301	GAAGACGCAT	CTAAACCCCC	NGTGAAGTAG	GTGACATTTT	TGTGGGNACT
351	CTTTTTGCAC	CNCGNTCTTA	GAGAGGCCAC	TTGANTTCAG	GNATGGCTGC

wherein N = A, C, G or T.

TBLASTIN of dBest

Query = Cucumber Thiolase 67696 (462 letters)

	reading	High	Probab.
	Frame	Score	P(N) N

Sequences producing High-scoring Segment Pairs:

gnl/dbest/21210 T04395 cDNA Lambda-PRL2 A. thaliana H... +2 291 7.1e-38 2

Example 4: Construction of Antisense vectors, pJO5F, pJO35H, pJO39H, and pJOFAF

Liquid *E. Coli* were grown in standard LB media containing the appropriate antibiotics at 200 RPM on rotary shakers at 37°C and *E. coli* colonies were grown on standard LB-agar plates in an incubator at 37°C [Protocol and Applications Guide, 2nd ed. Promega]. Liquid *agrobacterium* cultures were grown in standard LB media containing the appropriate antibiotics at 220 RPM on rotary shakers at 28°C and *Agrobacterium* colonies were grown on standard LB plates containing the appropriate antibiotics in an incubator at 28°C.

For all manipulations requiring DNA amplification in *E. coli*, either strain DH5 α (*suppE44*, Δ *lacU169* [ϕ 80*lacZ* Δ M15], *hsdR17*, *recA1*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*) or XL1-Blue (*suppE44*, *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*, *lac'*, *F'* [*proAB*⁺, *lacI'*, *lacZ* Δ M15, *Tn10*(*tet*)]) were used as recipients of recombinant plasmids. Plant transformation using the binary vector system was conducted using *Agrobacterium tumefaciens* strain GV3101 containing a *vir*⁺ Ti-plasmid lacking the t-DNA region.

The cloning of cDNA fragments into pJO530 (pBIN19 derivative, [Bevan et al. (1984) Nuc. Acids Res. 12, 8711]) and the subsequent transformation of *E. coli* and *Agrobacterium* were

performed according to standard procedures [Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed.]. cDNA fragments in λ -Ziplox were excised by restriction digest of the plasmid with XbaI and Sal I. The fragments were gel-purified, removed from the agarose (GeneCleanTM, BIO 101 Inc. La Jolla, CA), and ligated into pJO530, which was previously linearized by restriction digest with XBA I and Sal I. Figure 4A shows generic maps for vectors pJO5F, pJO35H, and pJO39H where the cDNA fragment was originally cloned into λ -Ziplox. The cDNA fragment in λ -ZAP II was excised by restriction digest of the plasmid with Kpn I and XbaI. The fragment was gel-purified, removed from the agarose, and ligated into pJO530, which had been linearized by restriction with Kpn I and Xba I. Figure 4B shows the plasmid map of vector pJOFAF. For all vectors proper insertion of cDNA fragments in the antisense orientation was determined by restriction digest analysis and sequencing of the insert. Sequencing of cDNA inserts in pJO530 was conducted in both directions using primers specific to regions flanking the multiple cloning site-pJO530-5', 5'-TGA ACTCTATCATTGATAG-3'; pJO530-3', 5'-AGTAACGGGTGATATATTCA-3'.

Example 5: Construction of "Double" Antisense Vector, pJO530

The construct, pJO530 (Figure 5) was made by inserting cDNA 5F into pJO35H. The cDNA fragment 5F was prepared as described in Example 4 and blunt-ended by filling the recessed termini using Klenow. The vector pJO35H was prepared by restriction digest with Sma I and the ends dephosphorylated with calf-

intestinal alkaline phosphatase. After repurification of the cDNA and linearized vector, cDNA 5F was ligated into pJ035H and an aliquot used to transform *E. coli* XL1-blue cells. Plasmid DNA was isolated from selected transformants showing resistance to kanamycin and checked for the presence of both inserts using a combination of double and triple restriction digests using Kpn I, Xba I, and Sal I. The proper orientation of 5F was determined by sequencing using primer pJ05305'.

Example 6: Production of Transgenic Plants

Arabidopsis plants to be used for transformation were grown in growth chambers at 20°C under a constant illumination of 70 $\mu\text{EM}/\text{m}^2$ with 12 plants per 3.5'' pot. Approximately 3-4 weeks after seed sowing the primary bolts of the plants were removed to facilitate growth of multiple secondary bolts. Plants were transformed by vacuum infiltration (Bechtold et al., Comptes rendus de L'Academie des Sciences Serie III Sciences de la Vie (1993) 316: p. 1194) 4 to 8 days after removal of the bolts.

Large liquid cultures of *Agrobacterium* to be used for transformation were prepared as specified in Example 4 above and transferred to infiltration media (1/2 MS salts [Murashige and Skoog (1962) *Physiol. Plant.* 15, 473], 1x Gambourg's B5 vitamins, 5% sucrose, and 0.044 μM benzylamino purine), by centrifugation and resuspension to an OD_{600} of 0.8. Vacuum infiltration was completed by immersing the pots upside down into a beaker containing the *Agrobacterium* suspension, which was then placed in a vacuum dessicator. A vacuum was applied for a duration of

either 2 or 5 minutes after the initial intense bubbling - of solution moving into the soil - had subsided. The pots of vacuum infiltrated plants were then returned to the growth room. Plants (T1-generation) were allowed to grow without further manipulation except for watering when necessary. Seed (T2 generation) was collected approximately 2 to 2 1/2 months after vacuum infiltration.

Transformed seed were selected by vigor of growth on 1/2 MS-agar plates (described in Example 1) containing 30 μ g/ml hygromycin. Resistant plantlets (T2 plants) were transferred to soil and allowed to grow as described for the T1 generation plants. When the plants were large enough, DNA was extracted from individual plants as subjected to Southern analysis using the cDNA as a probe [Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed] to determine if the cDNA had been incorporated into the genome and in what numbers of copies (Figure 6). Plants, selected for their resistance to hygromycin were found to have one to multiple copies of the cDNA.

Example 7: **Dependence of ACOX Antisense Seedling Growth on Exogenous Carbohydrate**

Seedlings antisense in ACOX show reduced post-germinative root growth compared to Col-0 when germinated and grown on media not supplemented with sucrose, as would be expected for those disrupted in β -oxidation. Approximately 50 T3-generation seed from various antisense lines produced from constructs pJ05F and pJ035H were prepared and plated (as described in Example 1) in petri dishes containing 1/2 MS agar-media with or without 20 mM

sucrose. Five days after plating, root lengths of all seedlings were measured and averaged for each antisense line. The data was analyzed as the ratio of average root length for seedlings grown without sucrose to that of seedling supplemented with sucrose (Figure 7A). A ratio lower than that for Col-0 indicates reduced growth. The average root length for antisense seedlings was similar to that for Col-0 in the presence of sucrose.

The trait of reduced post-germinative growth was shown to be heritable through the corresponding root growth analysis of the progeny of ACOX antisense line 35H3-9. Approximately 150 seed from the T3 generation of this antisense line - a line exhibiting drastically reduced LCOX activity (see Example 7) - were germinated and grown on 1/2 MS agar plates containing 20 mM sucrose and 30 μ g/ml hygromycin. Thirty-three seedlings were selected and transferred to soil. Of the thirty-three seedlings, six survived to maturity and produced seed. In each case, the antisense seedlings showed a reduced capacity for growth compared to Col-0 (Figure 7B).

Example 8: Reduced ACOX Activity in ACOX Antisense Lines

Groups of ACOX seedlings used for ACOX activity analyses were prepared and extracted as specified in Example 2. Several lines exhibited reduced ACOX activity either in all three enzymes or in LCOX (Figure 8A). There was a general correlation among those seedlings exhibiting reduced post-germinative growth and reduced ACOX activity. One line, that exhibiting only reduced LCOX activity, was chosen for LCOX analysis in T4 progeny (see

Example 6). Seedlings from the T4 progeny also exhibited reduced LCOX activity (Figure 8B).

Example 9: **Induction of Fatty Acid Catabolism Genes in Developing Seeds of Brassica Napus Genetically Engineered to Produce the Novel Fatty Acid Lauric Acid**

These plants have been genetically modified to produce lauric acid in seeds, either free or in lipids, by expressing the California Bay medium-chain acyl-carrier protein thioesterase (MCTE) under the control of the seed-specific napin promoter as described by [Voelker et al. supra] herein incorporated by reference. Plants expressing MCTE in vegetative tissues was accomplished through use of the cauliflower 35S promoter. Such plants exhibit high expression of the MCTE, but little or no accumulation of medium-chain fatty acids, especially in vegetative tissues.

These seeds for the laurate-producing Brassica plants were obtained from Michigan State University. Dry Brassica seeds were imbibed in H₂O and kept at 4°C for at least 24 hours, but not longer than 4 days. Four seeds were sown per 12'' pot containing compost/vermiculite (2:1). Plants were grown at 20°C under constant illumination of 70 $\mu\text{E}/\text{m}^2$, well watered, and given fertilizer supplements weekly. The first flowers appeared approximately 6 weeks after sowing. Flowers were self-pollinated by hand and affixed with a label specifying the date in order to normalize morphological staging of the developing seed. At the specified times seeds were harvested from the maturing pods. RNA extraction and Northern blot analysis ICL expression, a gene marker for fatty acid catabolism, were conducted according to

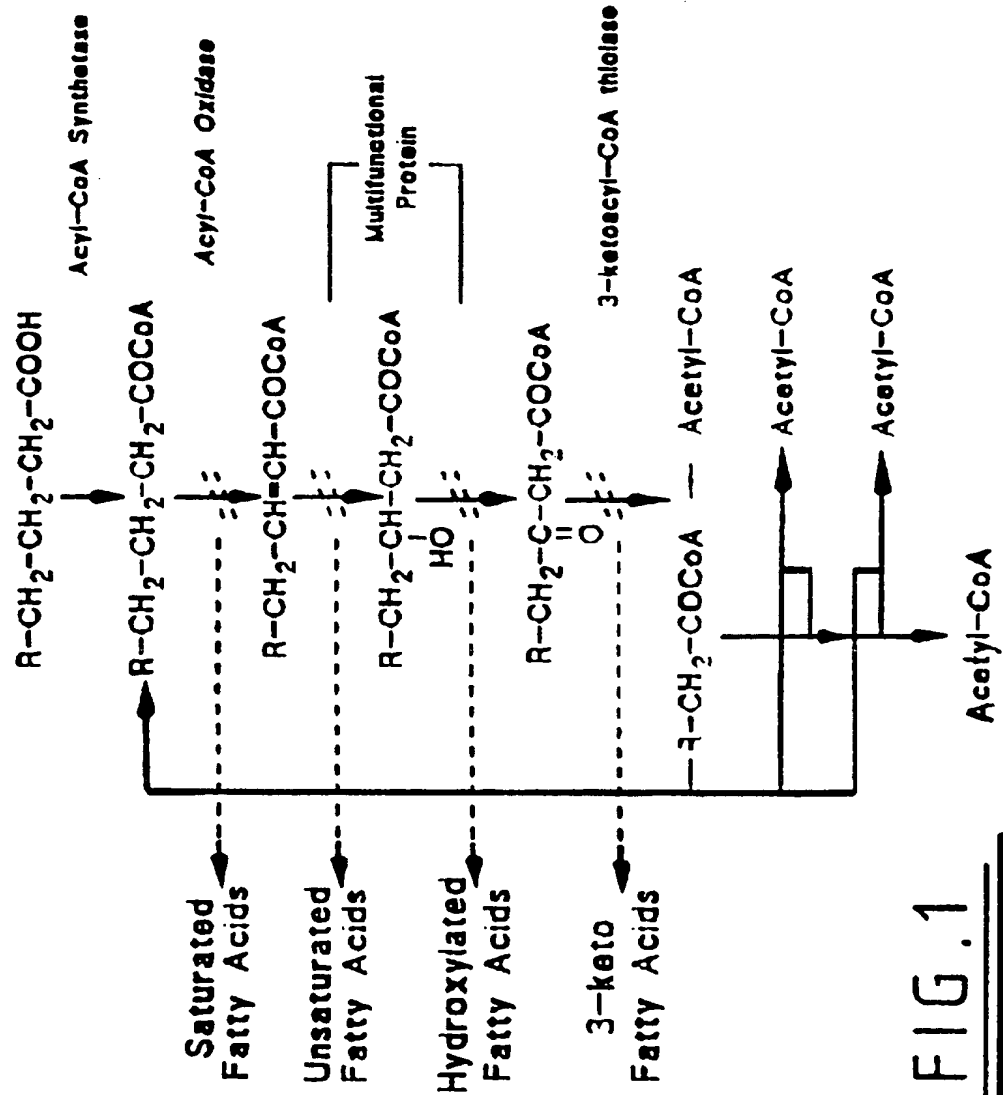
standard procedures. A significant induction of expression of the ICL gene was observed in the transgenic seeds compared to those from non-genetically modified *Brassica* plants (Figure 9). This demonstrated that the excess production of fatty acids leads to an increase in the machinery responsible for their catabolism.

Claims

1. A nucleotide sequence comprising a transcriptional regulatory sequence, a sequence under the transcriptional control thereof which encodes an RNA which consists of a plurality of subsequences, characterised in that at least two of the RNA subsequences are anti-sense RNA's to mRNAs of proteins having an enzymic activity in the peroxisomal β -oxidation of fatty acids in plant cells.
2. A nucleotide sequence according to claim 1 optionally including a sense RNA subsequence encoding for a marker protein.
3. A nucleotide sequence according to claim 1 or 2 wherein the sequence under the transcriptional control of the regulatory sequence encodes an antisense RNA which comprises at least two subsequences having the sequences of anti-sense RNA's to mRNA's selected from the group acyl-CoA oxidases, multifunctional protein and 3-ketoacyl-CoA thiolase or fragments thereof.
4. A nucleotide sequence which is complementary to one which hybridises under stringent conditions with the sequence of any one of claims 1 to 3.
5. A DNA construct comprising the nucleotide sequence of claims 1 to 4.

6. A biological vector comprising the nucleotide sequence according to claims 1 to 4 or the construct of claim 5.
7. Plant cells containing the nucleotide sequence according to any one of claims 1 to 4, the construct of claim 5 or the vector according to claim 6.
8. Plants comprising cells containing a nucleotide sequence according to claim 1 or claim 5, the progeny of said plants which contain said nucleotide sequence stably incorporated and heritable in a Mendelian manner, and/or the seeds of said plants or such progeny.
9. Use of the sequence according to any one of claims 1 to 4 the construct of claim 5, or the vector according to claim 6 in the production of plant cells having an under-expression of at least one enzymic protein of β -oxidation.
10. Use of the sequence according to any one of claims 1 to 4, the construct of claim 5, or the vector according to claim 6 in the production of morphologically normal whole plants having an under-expression of at least one enzymic protein of β -oxidation.
11. A method of inducing an under-expression of an enzymic protein of β -oxidation in plant cells comprising introducing into such cells a nucleotide sequence according to any one of claims 1 to 4, or a construct according to claim 5, or the vector according to claim 6.

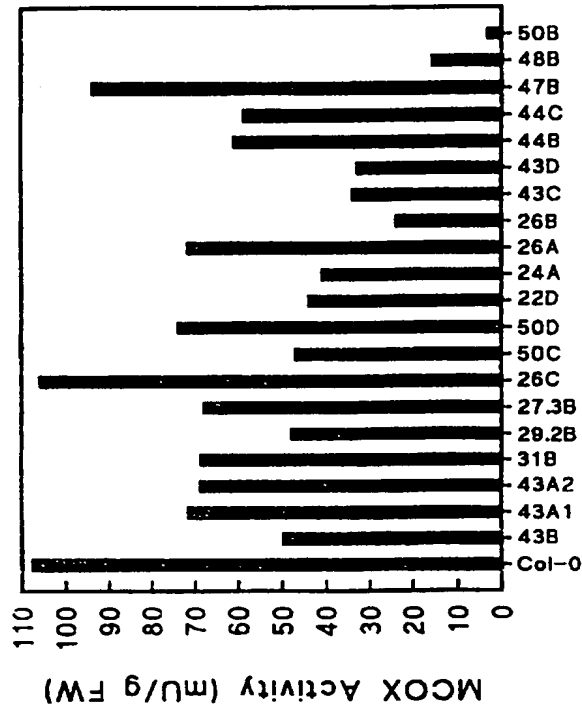
12. A method of inhibiting the production of at least one enzyme in a plant cell comprising introducing into the said cell a nucleotide sequence comprising a transcriptional regulatory sequence and a sequence under the transcriptional control thereof, which sequence encodes an RNA which consists of a plurality of subsequences, characterised in that the RNA subsequences are antisense RNA's to mRNA's of proteins having an enzymic activity in the peroxisomal β -oxidation of fatty acids in plant cells.
13. A method according to the preceding claim, wherein at least two of the subsequences is in an antisense configuration with respect to the coding sequence in the host cell mRNA which encodes the said proteins.



151

Pathway of β -oxidation showing end-products formed from a reduced or missing enzymatic activity (broken lines and arrows).

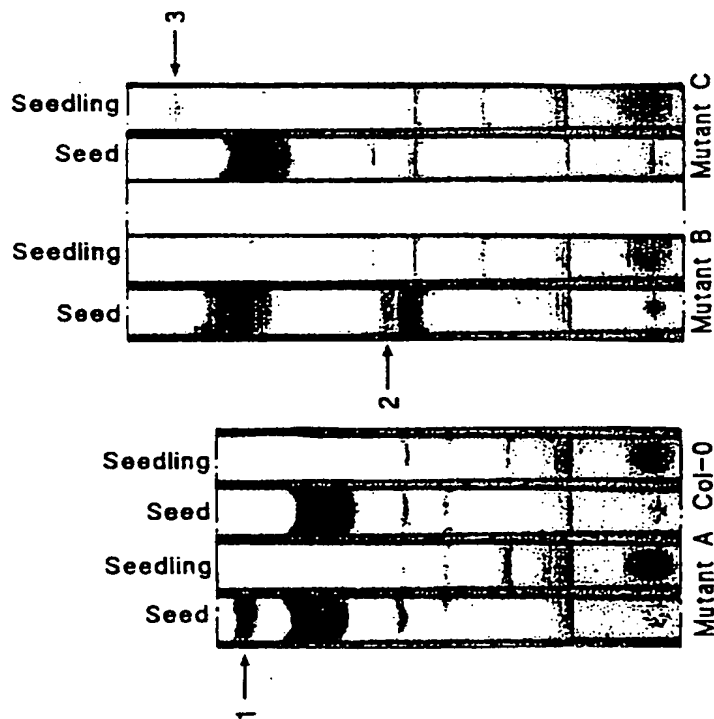
ACOX activities in sucrose-dependent mutants



LINE

FIG. 2B

Lipid profiles of seeds and seedlings of sucrose-dependent mutants



- 1 - New lipid in dry seed.
- 2 - New lipid in dry seed.
- 3 - Lipid accumulating during germination

FIG. 2A

ACOX activities in seedlings grown with (●) and without (○) sucrose

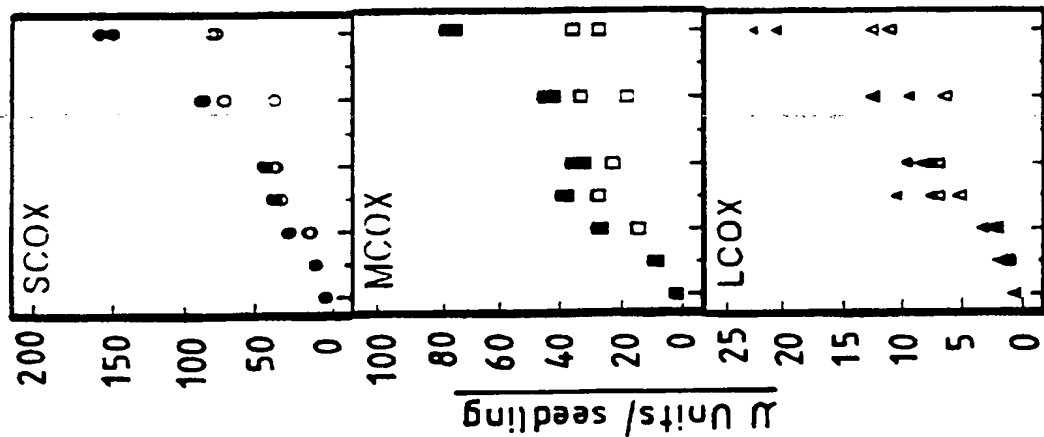


FIG. 3(a) DAYS after plating

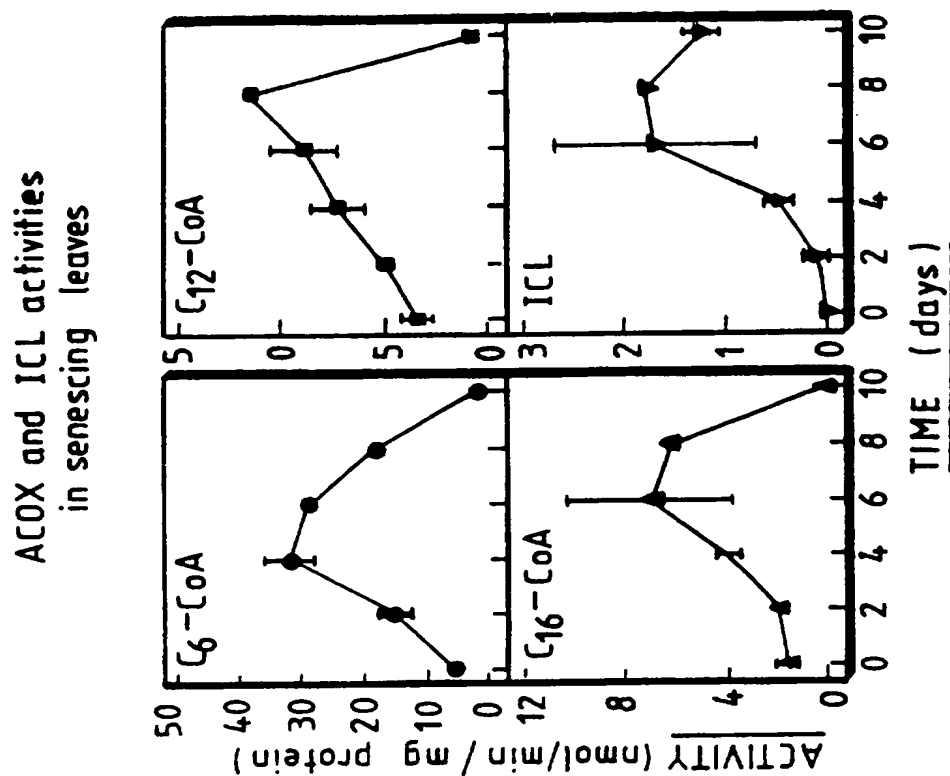


FIG. 3(b)

Generic map of plasmid vectors pJ05F, pJ035H and pJ039H

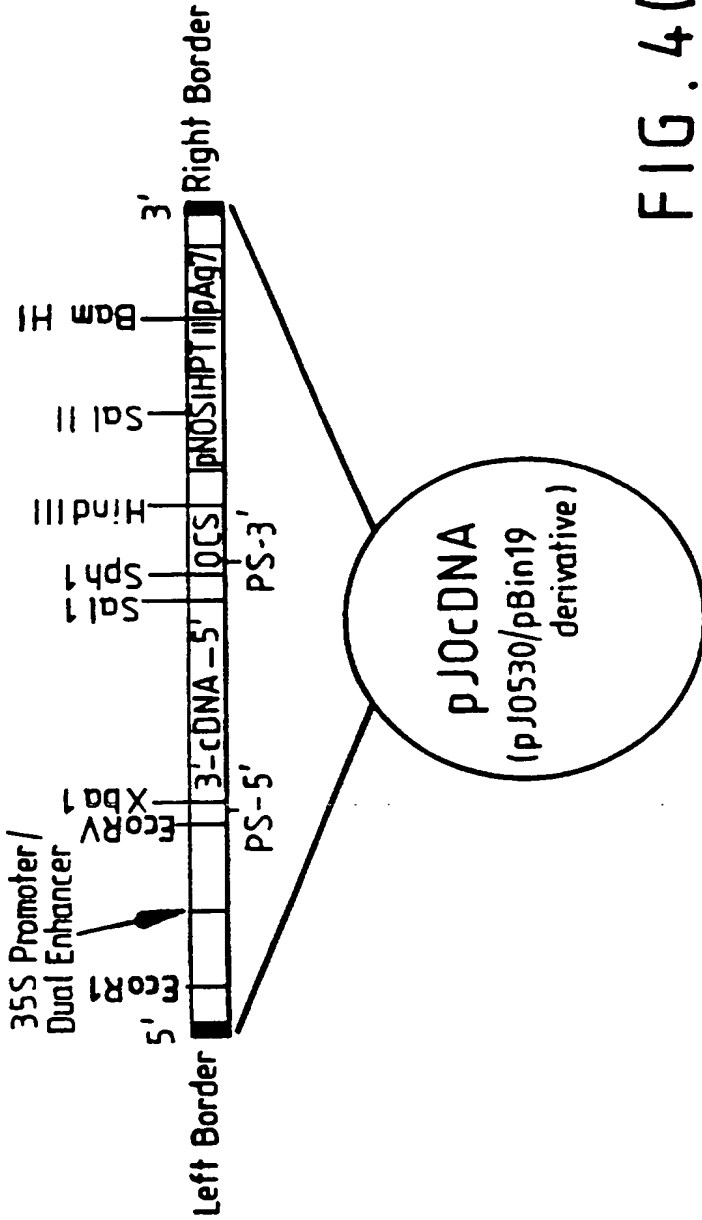


FIG. 4(a)

cDNA = 5F, 35H, or 39H
OCS — Octopine Synthase Terminator
pNOS — Nopaline Synthase Promoter
HPT II — gene conferring resistance to Hygromycin
PS-5' & PS-3' — Primer sites

Map of Plasmid vector pJOFAF.

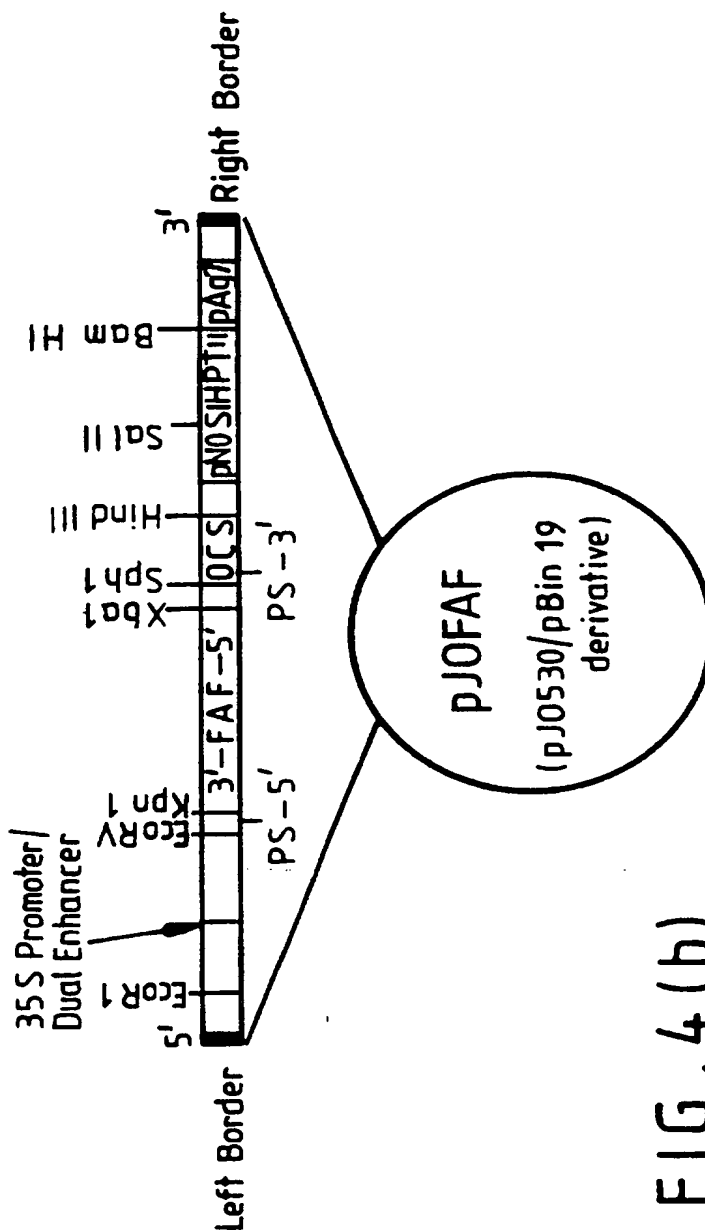


FIG. 4(b)

OCS - Octopine synthase terminator
 pNOS - Nopaline synthase promoter
 HPTII - gene conferring resistance to Hygromycin
 PS-5' & PS-3' - Primer sites

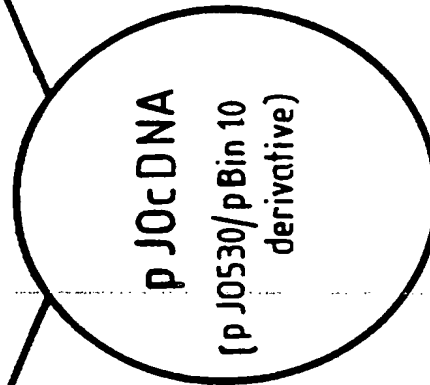
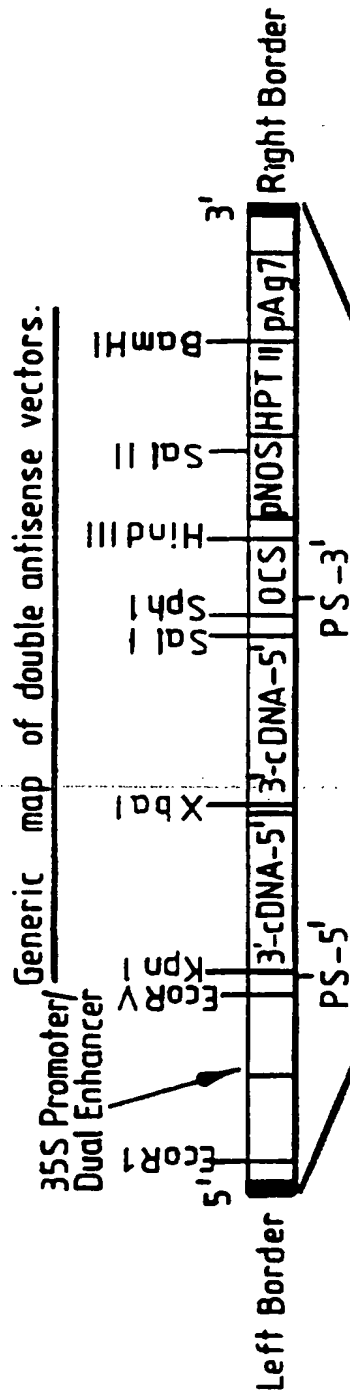


FIG. 5

OCS—Octopine synthase
pNOS—Nopaline Synthase Promoter
HPT II—gene conferring resistance to Hygromycin
PS-5' & PS-3'—Primer sites

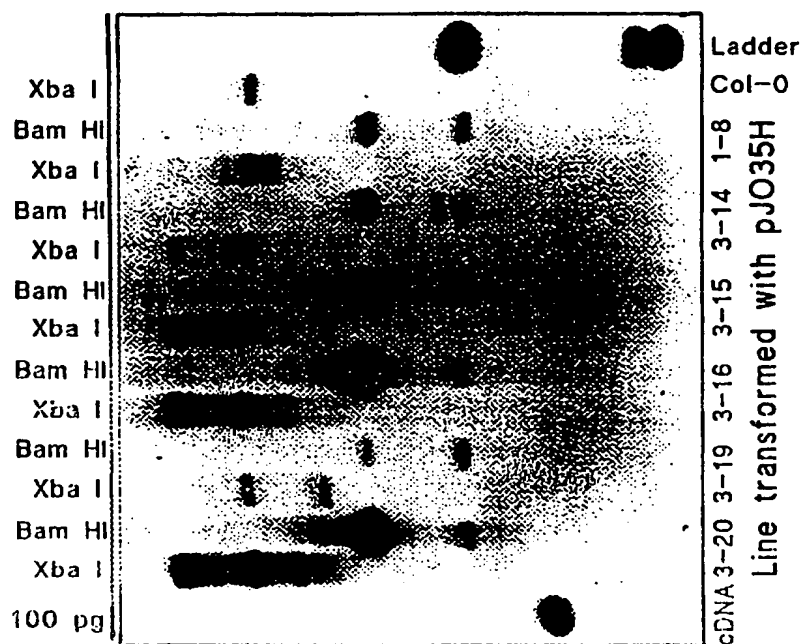


FIG. 6(b)

Southern analysis of ACOX antisense plants.

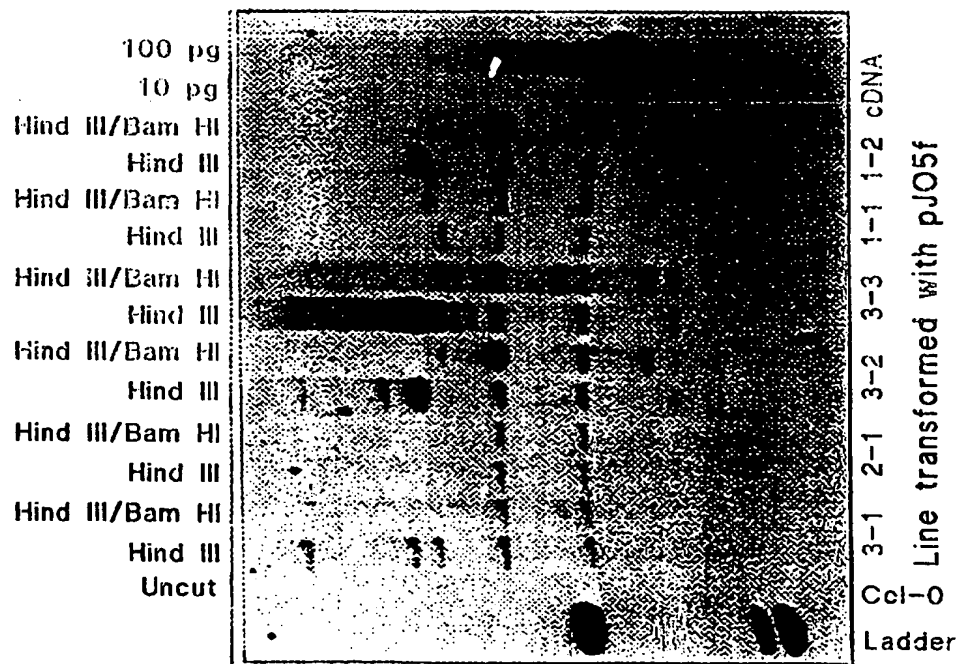


FIG. 6(a)

Effect of antisense on seedling root growth

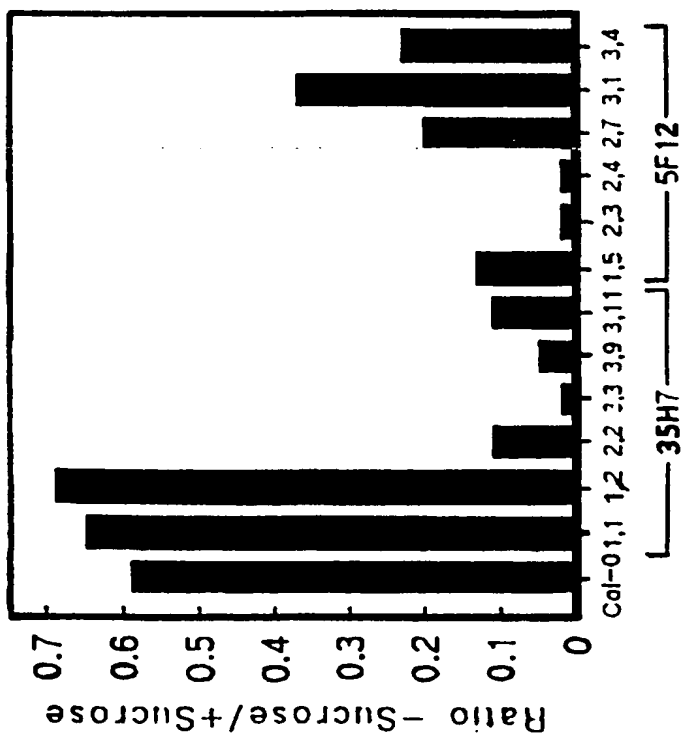


FIG. 7(a)

Effect of ACOX antisense on seedling emergence

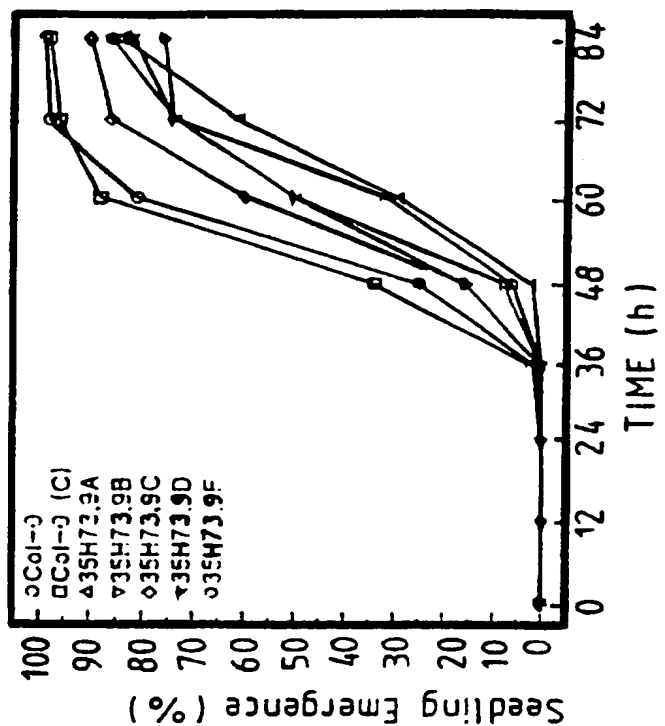


FIG. 7(b)

ACOX activities in antisense seedlings
transformed with p J035H (I3-generation)

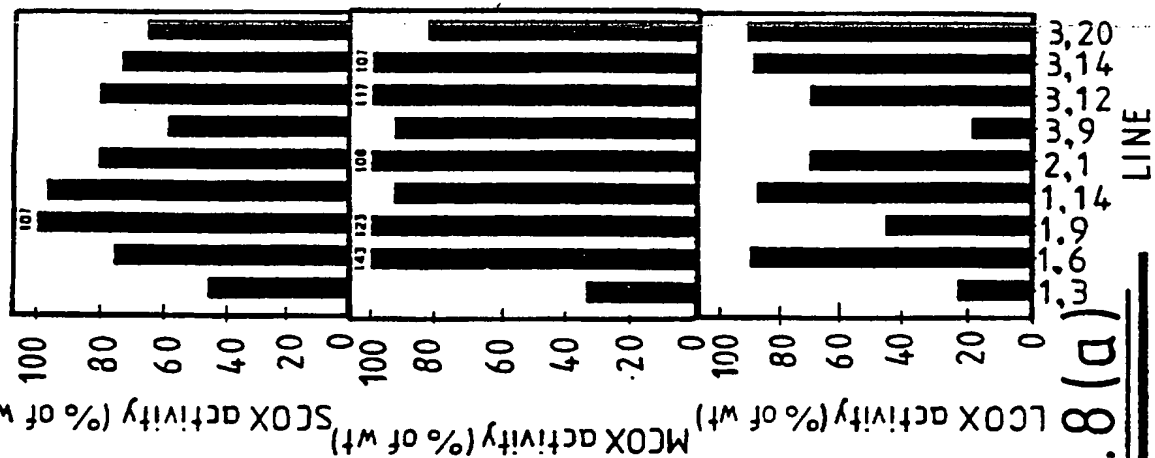


FIG. 8(a)

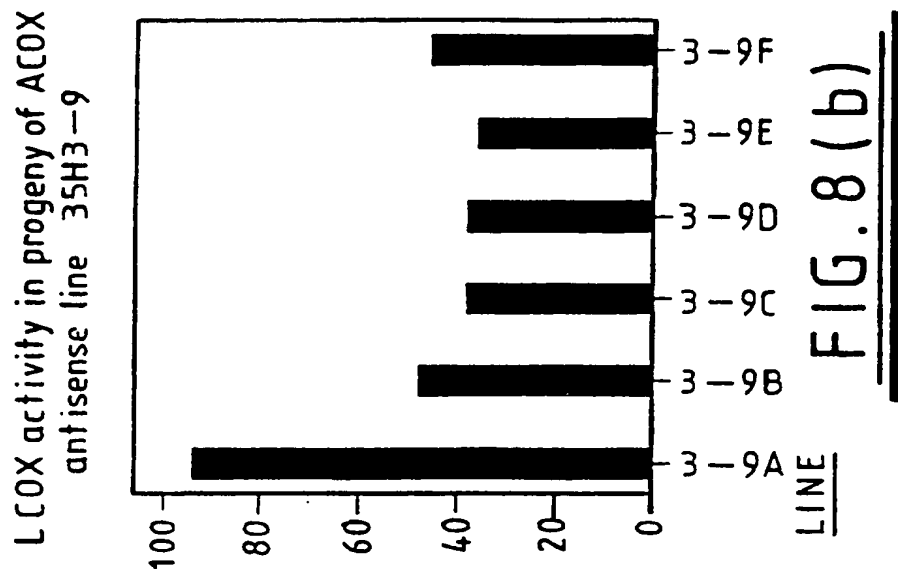


FIG. 8(b)

Induction of ICL expression in developing seeds
of Brassica napus overexpressing MCTE.

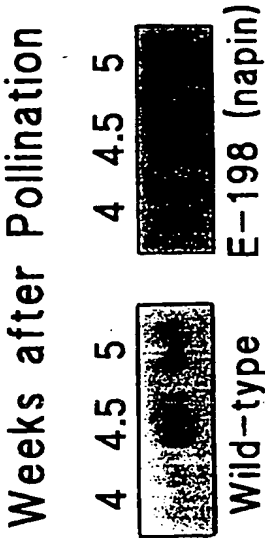


FIG. 9

INTERNATIONAL SEARCH REPORT

Inter national Application No
PCT/GB 97/01321

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/53 C12N15/54 C12N15/82 C12N5/10 C12P7/64
A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PHYTOCHEMISTRY, vol. 40, no. 3, 1995, pages 657-660, XP002037987 HOOKS M. ET AL.: "Regulation of acyl-CoA oxidases in maize seedlings" see the whole document ---	1-13
A	PLANTA, vol. 198, 1996, pages 46-53, XP002037988 ECCLESTONE V. ET AL.: "Medium-chain fatty acid biosynthesis and utilization in Brassica napus plants expressing lauroyl-acyl carrier protein thioesterase" cited in the application * see the whole document, esp. p.51 l. par.- p.52 * -----	1-13

☐ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

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- *&* document member of the same patent family

Date of the actual completion of the international search

19 August 1997

Date of mailing of the international search report

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